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Directly labeled DNA probes using fluorescent nucleotides with different length linkers

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ABSTRACT

Directly labeled fluorescent DNA probes have been made by nick translation and PCR using dUTP attached to the fluorescent label, Cy3, with different length linkers. With preparation of probes by PCR we find that linker length affects the efficiency of incorporation of Cy3-dUTP, the yield of labeled probe, and the signal intensity of labeled probes hybridized to chromosome target sequences. For nick translation and PCR, both the level of incorporation and the hybridization fluorescence signal increased in parallel when the length of the linker arm is increased. Under optimal conditions, PCR yielded more densely labeled probes, however, the yield of PCR labeled probe decreased with greater linear density of labeling. By using a Cy3-modified dUTP with the longest linker under optimal conditions it was possible to label up to 28% of the possible substitution sites on the target DNA with reasonable yield by PCR and 18% by nick translation. A mechanism involving steric interactions between the polymerase, cyanine-labeled sites on template and extending chains and the modified dUTP substrate is proposed to explain the inverse correlation between the labeling efficiency and the yield of DNA probe synthesis by PCR.

INTRODUCTION

Since its invention, (1,2) the technique of *in situ* hybridization (ISH) has become increasingly important in biological and diagnostic research (3). Fluorescent labels have gradually replaced the radioisotopic ones due to safety concerns, high spatial resolution, long shelf life, short detection time and simultaneous detection of multiple sequences in single cells. DNA probes are commonly labeled by nick translation (3, 4, 5). In recent years polymerase chain reaction (PCR) has been introduced to synthesize DNA probes (6, 7).

Most fluorescent *in situ* hybridization (FISH) experiments have been done using indirectly reagents, such as avidins and antibodies, for visualization of probe signals (3, 4). This requires a post-hybridization labeling step, which is time consuming and may increase background fluorescence. Additional signal amplification steps are possible to increase the intensity of the signal (3, 4), but again, background fluorescence may increase. If fluorophores are directly attached to DNA, extra steps can be eliminated and signal-to-background ratio may be increased even if the overall fluorescence intensity is somewhat reduced compared with indirect labeling.

In our previous study (8), Cy3 covalently conjugated through a short linker to dUTP was shown to be incorporated into DNA by nick translation and PCR. Other investigators have shown that if a linker molecule is inserted between biotin and a nucleotide molecule for nick translation, the labeling efficiency and hybridization detectability of Southern blotting experiment are improved (9, 10). Here we report systematic study of linker length on DNA probe labeling efficiency, yield of the PCR synthesis, and FISH signal intensity using Cy3 linked to dUTP.

MATERIALS AND METHODS

Modified dUTPs

Cy3.29.OSu synthesized as described previously (11), contains 6 atoms between the fluorophore ring system and the active ester group. For addition of 7 atoms to the linker, a mixture of 100 mg Cy3.29.OSu and 18 mg 6-amino caproic acid (Aldrich) was dissolved in 15 ml sodium bicarbonate buffer (0.1M, pH = 9.4) and stirred at room temperature overnight. The product Cy3.29-13-OH (13 denotes the number of atoms between fluorophore ring system and carboxylic group) was isolated by reversed-phase (RP) chromatography (C-18) using water-methanol mixture as eluent and dried down.

Cy3.29-13-OH was activated to succinimidyl ester, Cy3.29-13-OSu, by methods used to prepare Cy3.29.OSu (11). The same procedure was repeated to conjugate another 6-amino caproic acid with Cy3.29-13-OSu to obtain Cy3.29-20-OH and to form the activated Cy3.29-20-OSu. The chemical structures were confirmed by ^{1}H NMR. Cy3-x-dUTP (x = 10, 17, 24) was synthesized as described (8) The concentrations were determined by absorbance measurement at 550 nm using the extinction coefficient of Cy3, 150,000 L/mol.cm (11).

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PCR

PCR was generally carried out according to procedure of Yu (8). The PCR reaction mixture contains 1.5mM of $MgCl_2$.

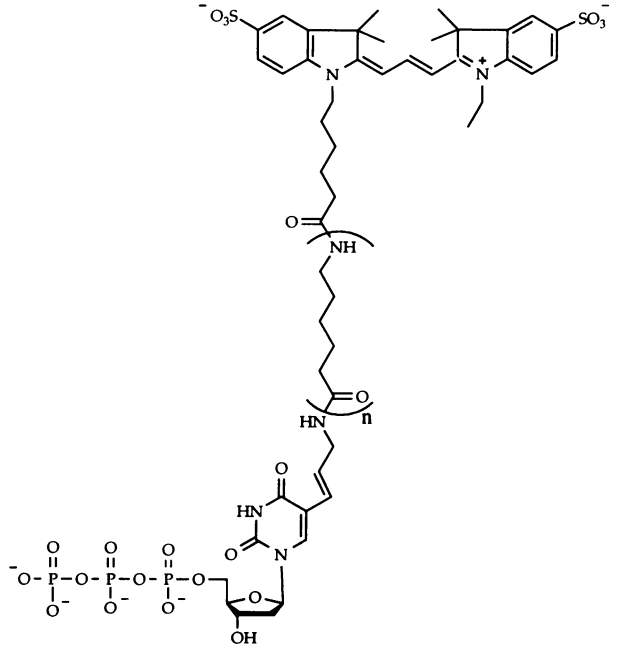
When a certain amount of modified dUTP was used instead of dTTP, the total concentration of modified dUTP and dTTP was kept the same as the concentration of each of other 3 nucleotides because the maximum fidelity of polymerization requires a balanced concentration of each of the 4 dNTPs (12).

The template for all nick translation and PCR experiments was a 900bp chromosome 1 insert (ATCC cat. no. 59863) in plasmid DNA. The primers were two ends of the vector (8), which leads to amplification of the entire 900bp insert. For quantification of DNA synthesis and Cy3 incorporation, the 5'-end of each primer was labeled with Cy5.18.OSu as previously described (13).

PCR was performed in a DNA Thermal Cycler (Perkin Elmer Cetus). The temperature cycling parameters were similar to the ones used by Ried *et al.* (14). After an initial denaturation at 94°C for 4 min, 30 cycles (except cycle number study) of PCR were carried out with denaturation at 94°C for 1 min, annealing at 52°C for 2 min and extension at 72°C for 3 min. Prep-A-Gene method (BioRad) was used for purification to remove unreacted Cy3-x-dUTP and excess Cy5 labeled primers. The molecular weight of PCR product was examined using a 1% agarose gel with 0.5 μg/ml ethidium bromide.

In order to obtain the labeling efficiency and the yield of PCR, the product was dissolved in 140 μl sterile water for fluorescence measurement of Cy3 and Cy5 with a Spex Fluorolog 2 spectrofluorometer (Spex Industries, Inc.). Calibration of fluorescence intensities was accomplished by measuring the fluorescence of known concentration of Cy3 and Cy5, both in a purified carboxylic acid form. Using the calibration data, the Cy3 and Cy5 emission intensities of labeled DNA were converted to Cy3 and Cy5 molar concentrations. Since Cy5 concentration is identical to the concentration of DNA (use of Cy5 labeled primers), it was possible to calculate from fluorescence measurements the amount of DNA produced in PCR, which was taken as the yield of the reaction. The Cy3/Cy5 molar ratio corresponds to the number of Cy3 molecules per DNA chain of PCR product. Assuming 1/4 of bases of this DNA are thymine, the Cy3/Cy5 molar ratio divided by 225 ($\frac{900b}{4}$) can be used as an estimate of the degree of substitution of Cy3-U (henceforth U*) for T in product DNA, defined as S_{Prod}. Thus, $S_{Prod} = \frac{U^*}{U^*+T}$. The quantum yield (Q. Y.) of Cy3-labeled DNA was determined as previously described (15).

Since the 900 bp labeled DNA probes are too long for optimal FISH, the PCR product was incubated with 1/350 unit of DNase I in nick translation buffer at 37°C for 5 min. A 1% agarose gel with 0.5 μg/ml ethidium bromide was used to check the chain length (Optimal length is 200–400 bp (3, 4).). The DNA fragments were ethanol-precipitated and reconstituted in 150 μl sterile water. The ratio of substitution and amount of DNA fragments were determined again by absorption spectroscopy with a HP 8452 diode array spectrophotometer (Hewelett-Packard). The average extinction coefficient of a base in double stranded DNA at 260 nm was 6500 L/mol.cm (16).



n = 0 Cy3-10-dUTP

n = 1 Cy3-17-dUTP

n = 2 Cy3-24-dUTP

Figure 1. Chemical structures of Cy3-modified dUTPs.

Labeling probes by nick translation

Nick translation was carried out under sequential reaction conditions found to be optimal for cyanine-modified dUTPs (8).

In situ hybridization and signal analysis

A common procedure was used for FISH and an imaging microscope system was used to quantify hybridization signals (8). For each slide, at least 24 FISH signals were collected. Hybridization signals were analyzed by 'spot analysis' software. First an algorithm was used to define the boundaries of each hybridization spot. Then fluorescence signals within the spot were quantified and an average background measurement was made in an area outside of the spot boundary where the spot signal was no longer significant. Two parameters were used to describe the FISH signals. The net spot intensity (NSI) was the sum of all pixel intensities within the spot minus the background net intensity of an area equivalent to the spot. The signal-to-background ratio (S/b) is the NSI divided by the background intensity of an area equivalent to the spot.

RESULTS AND DISCUSSION

PCR and nick translation reactions vary from day-to-day depending on the activity of the enzyme and reaction conditions. Therefore experiments presented in the same figure or same table have been done in the same batch for maximum consistency. The same trends were observed for data obtained on different days

The chemical structures of Cy3-modified dUTPs (dU*TPs) are shown in Fig. 1. In this work we are concerned with optimizing the substitution efficiency and the yield of DNA probes directly labeled by PCR or nick translation with dU*TPs. The key variables under our control are the length of the linker arm between the ring structure of the fluorescent label and the uracil

ring, and the ratio of substitution of dU*TP for dTTP in the reaction mixture. We define the ratio of substitution of modified uracil for thymine i.e., $\frac{U^*}{U^*+T}$ in the reaction mixture and in the product as S_{Rxn} and S_{Prod}, respectively. Thus, the efficiency of labeling is quantified by S_{Prod} and a value of 1 means that all possible sites for incorporation of a thymine contain a modified uracil after nick translation or PCR.

The amount of incorporation of Cy3 into probe can be determined by two methods (see 'Materials and Methods' for details). In one method, an absorption spectrum of purified product yields the absorbance of nucleotide bases at 260 nm and the absorbance of Cy3 at 550 nm. The dye/base ratio can be calculated from this data with knowledge of the extinction coefficients. A second method involving measurement of fluorescence is useful only for PCR but it is more sensitive and requires less material. Primers labeled at the 5'-end with a different fluorophore, Cy5, are used in this method to determine the number of copies of DNA synthesized during amplification. The ratio of Cy3 fluorescence to Cy5 fluorescence allows calculation of the number of Cy3 molecules incorporated per copy of probe DNA. Quantification of S_{Prod} and probe yield by fluorescence measurement requires that the fluorescence efficiency be independent of the linear density of Cy3 molecules on the probe and that the Cy5 molecule is not involved in energy transfer from Cy3 molecules on the probe. With 100% substitution of U* for T there should be an average of one Cy3 molecule every 4 bases. In our experiments, there is no indication of significant (>1.5×) fluorescence quenching at labeling densities up to 38% substitution of U* for T. And determined S_{Prod} relative order is not affected (data not shown). According to previous calculations, the energy transfer from Cy3 to Cy5 can be neglected because the length of the primer sequence that separates them (8). Thus S_{Prod} and the yield of labeled DNA determined by fluorescence measurement in this study should be reasonably accurate.

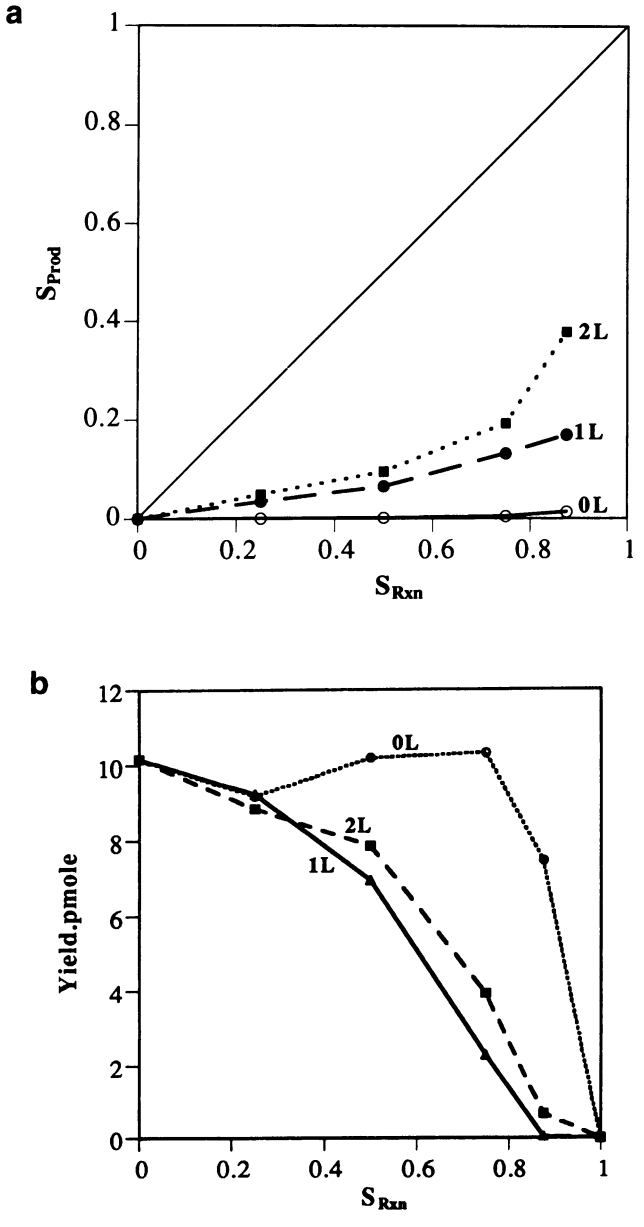


Figure 2. a. S_{Prod} vs. S_{Rxn} of PCR for modified dUTPs. We found that the high base concentration does not significantly improve the yield and higher base concentrations are known to cause lower fidelity of polymerization (12). Therefore a low base concentration, 60 mM, was used for all other PCR experiments. S_{Prod} and the yield were determined by fluorescence measurement. 0L = Cy3-10-dUTP; 1L = Cy3-17-dUTP; 2L = Cy3-24-dUTP. **b.** Yield vs. S_{Rxn} of PCR for modified dUTPs. The yield was determined by fluorescence measurement. Same samples as a.

Incorporation of Cy3-modified dUTP by PCR

The yield of Cy3-labeled probe generated by PCR comes to a plateau region after approximately 20 cycles. The yield of PCR product in a reaction using only dTTP and no dU*TP reaches a similar plateau. Although we have not tested thermal stability of dU*TP, this result shows that dU*TP is almost as stable as dTTP. Otherwise we will expect to see a much higher plateau of the reaction using only dTTP and no dU*TP. Because there was not much improvement in the yield after 30 cycles, all other PCR experiments were carried out at 30 cycles. S_{Prod} does not change with the cycle number.

The observation that the yield of probe using a 50:50 mixture of dTTP and dU*TP (S_{Rxn} = 0.5) is almost the same as the yield when dTTP alone is used indicates that under the right conditions incorporation of dU*TP does not interfere with the formation of complete PCR product. However, this does not imply that dU*TP is as good a substrate as dTTP. If the reactivity of the modified nucleotide is the same as natural nucleotide in PCR, there should be a linear relationship between S_{Prod} and S_{Rxn} with a slope of 1. In Fig. 2a, even the highest S_{Prod} is below the line with a slope of 1, which means that Taq polymerase favors the natural nucleotide over the modified nucleotide even under the best reaction conditions. The Taq enzyme normally shows not much preference for uracil over thymine (17). Therefore the fluorophore and linker must account for the reduced capability of dU*TP to act as a substrate.

The efficiency of labeling DNA with Cy3 in a PCR reaction is increased when S_{Rxn} is increased (Fig. 2a). Thus, with increased S_{Rxn}, the probability of substitution of dU*TP for T during incorporation is greater and leads to higher S_{Prod}. The increased linear density of Cy3 molecules on product that occurs with higher S_{Rxn} is also reflected in the decreased mobility of these PCR products on electrophoresis gels. It has been reported that the incorporated digoxigenin can cause the retardation of mobility of PCR products on the electrophoresis gel (18, 19).

Increasing the linker length also favors greater incorporation of dU*TP (Fig. 2a). It is possible that longer linkers reduce steric interactions between the Cy3 molecule and the polymerase−DNA complex thus making dU*TP with longer linkers better substrates.

Although the efficiency of incorporation of modified bases by PCR improves as linker length or S_{Rxn} increases, the yield of labeled probe generated by multiple PCR cycles is reduced, and when 100% dU*TP was used instead of dTTP, there is no amplification (Fig. 2b). We found that the longer extension time does not raise S_{Prod} and the yield actually decreases. This may be caused by the loss of enzyme activity during long incubation at 72°C. An extension time as short as 1 minute does not affect the labeling efficiency and yield.

Why does the yield of Cy3-labeled probe in a PCR reaction decrease when the labeling density increases? One explanation is based on the steric considerations. When more U* is incorporated into a DNA probe, there is a greater possibility of modified bases appearing in close proximity to one-another. In the next cycle, the labeled chain serves as the template. The presence of several Cy3 fluorophores in a small space on the template (or the template and extending chain) may inhibit the progression of the polymerase and lead to termination of the probe fragment. Since incomplete chains can not be used as templates in the next cycle, the yield is decreased. Finckh *et al.* noted a similar decrease in yield of labeled probe when the substitution of biotin modified dUTP was increased (19).

According to this model, the yield is reduced because of a high labeling density that sterically inhibits translation of the labeled template and that can be generated by either using a high S_{Rxn} or by using probes with longer linkers. However, there is a compensating effect that appears with longer linkers that reduce steric constraints from the fluorophores. This effect shows up in Fig. 2b. Since the labeling density for Cy3-24-dUTP is greater than for Cy3-17-dUTP when S_{Rxn} is in the range of 0.5−0.9 (Fig. 2a), it would be expected that the yield of reaction of Cy3-24-dUTP would be smaller . However, Fig. 2b shows that there is a greater yield of Cy3-24-dUTP than Cy3-17-dUTP over this range of S_{Rxn}. It is apparent that the longer linker facilitates chain extension even when the template is more heavily labeled. Therefore Cy3-24-dUTP is the best substrate for DNA probe labeling by PCR because it can give the highest labeling density with reasonable yield.

Incorporating Cy3-modified dUTP by nick translation

Table 1 shows that under optimal nick translation conditions S_{Prod} values for Cy3-17-dUTP and Cy3-24-dUTP are nearly equivalent but are about two-fold higher than for Cy3-10-dUTP.

Table 1. S_{Prod} and FISH result of DNA probes

	S_{Prod}	NSI (10^4)	S/b
NT.0L	0.11	0.5 ± 0.3	0.12 ± 0.05
NT.1L	0.18	1.5 ± 0.6	0.24 ± 0.07
NT.2L	0.18	1.2 ± 0.6	0.22 ± 0.07
PCR.0L	0.01	0.2 ± 0.1	0.07 ± 0.04
PCR.1L	0.14	1.7 ± 1.3	0.23 ± 0.11
PCR.2L	0.28	2.3 ± 1.6	0.29 ± 0.08

S_{Prod} was determined by absorbance measurement. PCR probes were the same as those for Fig. 3a after DNase digestion. NT = Nick translation; 0L = Cy3-10-dUTP; 1L = Cy3-17-dUTP; 2L = Cy3-24-dUTP. The FISH parameters are described in 'Materials and methods' section.

Our early study of nick translation with a different template (a SstI fragment) yielded similar results (20). This SstI fragment is a moderately repetitive sequence of 2.5 kb appearing in tandem arrays on human chromosome 19 and less frequently on chromosome 4 (21, 22). Others have reported that as the distance between biotin and nucleotide was increased, the labeling efficiency by nick translation first increased and then stayed constant or decreased (9, 10). Apparently steric interactions with the fluorophore on a shorter tether lead to less efficient incorporation of U* in the nick translation reaction.

Since a DNA probe made by nick translation is composed of extending fragments and residual fragments and only extending fragments can be labeled, overall S_{Prod} of a DNA probe depends upon both S_{Prod} of extending fragments and the ratio of extending fragments to residual fragments. In carrying out these reactions dU*TP completely replaces dTTP, so that in the extending fragments all T-sites contain U*. On the other hand, for PCR, the whole probe except for the short primer, can be labeled. Thus, with equally efficient incorporation by the polymerase, PCR should produce probes with an overall higher linear density of labeling. However, we have shown that there can not be complete substitution of dU*TP for dTTP in polymerase reaction. Even though S_{Rxn} is 1 in nick translation and S_{Rxn} is less than 1 in PCR, the optimal S_{Prod} of PCR is still higher than that of nick translation.

Fluorescence *in situ* hybridization (FISH) with Cy3-labeled probes

After having examined the labeling efficiency and yield of fluorescent DNA by PCR and nick translation, we now turn to the utility of such probes in FISH. Target DNA of chromosome 1 in interphase nuclei of HeLa cells was hybridized with Cy3-modified probes. FISH signals were quantified by image cytometry.

Table 1 shows that both the net spot intensity and the signal-to-background ratio of signals follows the same trend as S_{Prod} of probes. This means that the long linker does not seem to affect probe mobility and entry to the nuclei. Since the background is not increased as fast as the signal intensity, the fidelity of

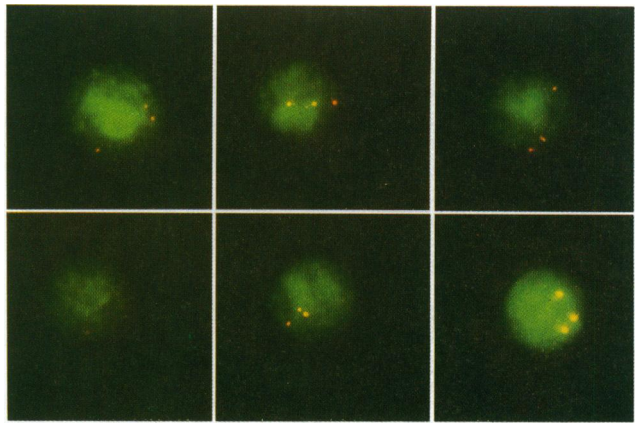


Figure 3. FISH images by different DNA probes. The upper images were obtained using probes made by nick translation. The bottom images were obtained using probes made by PCR. From left to right: Cy3-10-dUTP, Cy3-17-dUTP, Cy3-24-dUTP.

incorporating modified nucleotide is high enough to serve the purpose of preparing DNA probes . Thus improving probe labeling is a way to optimize FISH.

Since the FISH signal intensities obtained with PCR-generated probes are higher than those of probes made by nick translation (Table 1), and since PCR can label and amplify DNA probes simultaneously, PCR is a good labeling technique for preparation of directly labeled fluorescent DNA probes. Even higher labeling densities are achievable by PCR but at the price of low yield.

FISH images of 6 kinds of probe are shown in Fig. 3. The signal intensity of each image is close to the respective average intensity of each kind of probe.

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